NEW PICORNAVIRUSES, VACCINES AND DIAGNOSTIC KITS.

FIELD OF INVENTION

The present invention relates to new picornaviruses, proteins expressed by the viruses, antisera and antibodies directed against said viruses, antigens comprising structural proteins of said viruses, diagnostic kits, vaccines, use of said viruses, antisera or antibodies and antigens in medicaments, and methods of treating or preventing diseases caused by said viruses, such as Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

BACKGROUND OF THE INVENTION

Recently, a sudden death syndrome among Swedish orienteers has been observed. Of approximately 200 elite orienteers six died in myocarditis during 1989-1992 (1). Orienteering, aiming to find the fastest/shortest way between several checkpoints and often in forested areas, is exceptional with respect to environmental exposure. Thus it has been speculated, that the sudden deaths syndrome among orienteers is caused by a vector borne (rodent or arthropod) infectious agent.

It has now been shown in an epidemiological study that the incidence of deaths in myocarditis in northern Sweden tracked the 3-4 year population fluctuations (cycles) of bank voles (*Clethrionomys glareolus*) with one year time lag. Previously, it has been shown that cardioviruses, with rodents as their natural reservoir, can cause Guillain Barré Syndrome (GBS) in man, Diabetes Mellitus (DM) in mice and myocarditis in several species including non-human primates.

In addition to death in myocarditis it is also shown in the epidemiological study that the number of patients diagnosed with Guillain Barré Syndrome (GBS), and Diabetes Mellitus (DM) in northern Sweden tracked the 3-4 year population fluctuations of bank voles with different time delays.

Sven Gard and co-workers studied antibody prevalence to encephalomyelitis virus (EMCV) in Swedish normal population in the early 1950th (2). These studies found a surprisingly high antibody prevalence rate by hemagglutination inhibition test but no sera could be confirmed by neutralization test. These results were found puzzling at the time but could be explained by the presence of one or several related picornaviruses circulating in Sweden.

The fact that enterovirus have a large number of members and cardiovirus only two possibly three could reflect the true diversity of the two genus or only be the result of the amount of effort made to isolate new viruses from rodents as compared to isolating new enteroviruses from humans.

The Picornavirus family is presently divided into five genera (aphto-, entero-, hepato-, rhino-, and cardioviruses) (3). This taxonomy was initially based on morphological, physiological and serological properties as well as on the pathogenicity of the viruses. More recently, however, viruses have been characterized based on their genome sequence since it has been established that sequence data to a large extent coincide with the characterisation properties used previously (4,5).

The prototype virus in the cardiovirus genus is Theiler's murine encephalomyelitis virus (TMEV). Another member in this genus is encephalomyocarditis virus (EMCV). Vilyuisk virus, isolated from patients in Russia with degenerative neurological disease, is serologically related to TMEV but presently under consideration for being included as a third distinct member of the cardiovirus genus (6).

In nature, cardioviruses have a geographically widespread distribution and a large number of susceptible hosts with rodents as their natural reservoir. In addition to rodents, EMCV has been isolated from domestic pigs, elephants, lions, non human primates and man (7,8,9). Infection with TMEV and EMCV have provided excellent animal models for inducing myocarditis, DM and different neurological disorders such as demyelinating diseases resembling multiple sclerosis in mice (10-16). Other neurological or muscular disorders in which an infection is suspected to be the triggering factor and in which there is also an autoimmune component are Cardiomyopathia, Multiple Sclerosis (MS), Chronic Fatigue Syndrome (CFS), Myasthenia Gravis (MG), and Amyothrophic Lateral Sclerosis (ALS). It has never been established, however, that cardiovirus is a significant human pathogen, as disease in man most often has been described in case reports or as infection measured in sero-epidemiological surveys (7-17).

Thus, there may be other not yet identified picornaviruses circulating in the wild rodent population and occasionally infecting humans resulting in Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome, in genetically susceptible individuals.

The epidemiological link between important human diseases and small rodent abundance and what is previously known about picornavirus/cardiovirus motivated attempts to isolate novel picornaviruses from small rodents.

DESCRIPTION OF EXPERIMENTAL WORK AS BASIS FOR THE INVENTION

Trapping of animals

Small rodents were trapped at several locations in northern Sweden and transported live to the Swedish Institute for Infectious Disease Control in Stockholm, Sweden. Species, date and location of trapped animals were

recorded. Animals were bled using ether anaesthesia and killed. Organs were immediately removed and stored at -70°C until tested for presence of virus. A total of 53 *Clethrionomys glareolus* and 28 *Microtus agrestis* were tested for virus isolation.

Virus isolation

The isolation technique used in the present study was different from what is most often used. The cells used for isolation were kept for a minimum of two weeks and virus growths were detected by both CPE (cytopathogenic effect) and by staining the cells by a large number of human sera using IFT (immunofluorescense test). None of the new viruses presented herein would have been isolated using routine procedure for detecting cardioviruses/picornaviruses. They grow to lower titer and CPE develops slowly.

Saliva mixed with lung homogenate and faeces were analyzed separately from each animal. The material was inoculated into T25 flask of confluent BHK-21 cells. Cells were blind passed twice a week during two weeks. At the end of this period or earlier if signs of CPE occurred, cells were removed from the T25 flask by a rubber policeman, placed onto 10-well spot slides, air dried and acetone fixed. The cells were then stained with panels of human sera including 5 multiple scleroses patients, 5 patients recently diagnosed with DM and 5 athletes dying in myocarditis and bled at autopsy. All T25 flasks (saliva-lung and faeces separately) were tested individually by IFT using the complete panel of human sera at a 1:10 dilution.

Cells showing positive reaction by IFT using the human serum panels were selected for further analysis. This included inoculation intracerebrally into 1 day old suckling mice, serological characterisation and sequence analysis.

Antisera and ser logical pr cedures

Antisera to the virus isolates were raised in mice (NMRI), and Guinea Pigs (Dunkin Hartley). The animals were injected with a cell culture supernatant from

(BHK-21 cells) intraperitoneally and serum collected 4-6 weeks later. Preimmunization sera were tested individually while postimmunization sera were pooled from all infected animals.

An indirect immunofluorescense test (IFT), as described previously (18,19) was used to test antibody titres in immunized animals. Briefly, spot slides were prepared by incubating virus on Green Monkey Kidney (GMK) cells for 6-10 days. At sign of discrete CPE cells were removed from the flask by a rubber policeman and put onto the microscope slides, air dried, and fixed in cold (4°C) acetone and stored at -70°C. The titer was determined by incubating serum diluted in PBS in the slides at 37°C for 1 hour in a moist chamber, followed by a FITC conjugate ($F(ab')_2$ fragment of goat anti human IgG γ-chain specific, Sigma Immuno Chemicals (F-1641) or Rabbit anti mouse immunoglobulins Daco (F0313)) incubated as above.

Antibody titers to the viruses were determined by a modification of the Plaque reduction neutralization test (PRNT) as described by Earley et al (20). In the test, sera were serially diluted four-fold and mixed with an equal volume containing 80-100 plaque-forming units (pfu) of virus per 50 µl. The mixtures were then incubated at 37°C for 60 minutes, and 50 µl subsequently inoculated into each of 2 wells of a tissue culture plate containing confluent Vero cell monolayer. After adsorption for 60 minutes at 37°C the wells were overlaid with 0.5 ml of a 42°C mixture of 1 part 1% agarose and 1 part 2X basal Eagle's medium with Earle's salts, 17 mM Hepes buffer, 8% heated fetal calf serum, 100 U/ml penicillin, 100 µg streptomycin. The tissue culture plates were incubated at 37°C in a humidified 5% $C0_2$ atmosphere for 3-7 days. A second overlay (0.5 ml) containing neutral red stain (1:9000) was then applied and plaques were enumerated the following day. The plaque numbers were linearly extrapolated to 2-fold dilutions. An 50% reduction of plaques was used as the criterion for virus neutralization titers.

Electron microsc py

Cell culture media or brain tissue homogenates were examined by negative contrast electron microscopy (EM). A 10 µl droplet was incubated on Formvar/carbon-coated grids for one minute or alternatively, 0.5 ml samples were centrifuged for 30 minutes at 20,000 x g to remove cell debris and finally the supernatants were pelleted directly onto grids in a Beckman Airfuge for 10 minutes at 160,000 x g. Grids were stained with 2% phosphotungstate acid (pH 6.0) and examined in a Philips CM 100 electron microscope at a magnification of at least 46,000.

Sub. 8'

Sequence data

The isolates 87-012, 174F and 145SL were grown on the human lung carcinoma line A549 in 1600 cm^2 roller bottles. Full CPE was observed after 5-10 days. Supernate was filtered through 0.45 µM cellulose acetate filters (Costar) and the virus was pelleted at 20,000 g for 20 h at 4°C. RNA was isolated from the virus containing pellets using acid guanidinium thiocyanate as described (Chomczynski and Sacchi). Synthesis of cDNA was performed under standard conditions using 1 µg of RNA, AMV reverse transcriptase (Boehringer-Mannheim) and random 14 mer oligonucleotides as primers in a 20 µL reaction. Fragments of the viral 5'UTR were amplified using cardiovirus specific consensus primers: (sense) 5'-GGCCGAAGCCGCTTGGAATA-3' (SEM) and (antisense) 5'-GTGGCTTTTGGCCGCAGAG-3' (ATVEM), both primers modified after the EMCV2 and EMCV1 primers previously reported (Jongen et al. 1993. Ann. Reum. Dis. 52:575-578. Cardiovirus sequences were from Dr A. Palmenberg (personal communication). Amplification conditions were 30 cycles at: 94°C, 30 sec., 50°C, 30 sec, 72°C, 2 min. The amplified fragments were cloned into the pCRII T-vector (In-Vitrogen). The cloned viral sequences were sequenced using A Taq polymerase FS cycle sequencing kit and data was collected on a ABI Prism 310 sequencing machine using M13 -21 and M13 reverse primers (Perkin-Elmer). A 1.8 kb fragment extending from the 5'-UTR into the viral polyprotein sequences was obtained by PCR (polymerase chain reaction) amplification of cDNA from the 145SL isolate. The primers were:

(sense) 5'-ACAGTGCATTCCACAC-3' (SLJU1) or 5'-CCGCTCCACAATAGA-3' (SLJU2) and (antisense) 5'-GATCTCAGAC-3' (primer 118). The SLJU1 and SLJU2 primers are located immediately adjacent to one another and were chosen as consensus primers for the Ljungan isolates of the invention with as little homology as possible to the EMCV and TMEV groups of viruses. The amplification conditions were 30 cycles at: 94°C, 30 sec., 42°C, 1 min , 72°C 2 min. The antisense primer 118 yielded similarly sized PCR products with either the SLJU1 or SLJU2 as sense primers, but none of the primers yielded PCR fragments when used alone. The sequence of the primer 118 was previously published (Bauer, D., et al. 1993. Nucl. Acids Res. 21:4272-4280). The obtained 1.8 kb PCR fragment was cloned and sequenced as described above.

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RESULTS OF EXPERIMENTAL WORK

Three virus isolates were selected based on reaction with the human serum panels and showing a size and structure compatible with a picornavirus on EM. The first isolate was named Ljungan 87-012. Ljungan is a river in Medelpad county, Sweden where the animals were trapped.

The second and third isolate were designated Ljungan 174F and Ljungan 145SL, respectively.

All three isolates came from C. glareolus.

All three isolates killed suckling mice in 3-5 days.

The titer in mouse brain was 10^9 (approximately) while the cell culture titer was only 10^5 (approximately).

Electron microscopy

Virus particles, 27 nm in diameter, were spherical with the surface almost featureless and they appeared single or in small aggregates. In rare cases the stain penetrated the particles which made them look like empty shells.

Serological results

It was found after testing a number of different cell lines the Green Monkey Kidney cells were most suitable for making IFT drop slides for serology. The cross IFT data using mouse sera are seen in Table 1.

TABLE 1

Cross-IFT using virus infected GMK cells. Immune mice were titrated using 4 fold dilutions starting at a 1:10 dilution.

	VIRUS		
	87-012	174F	145SL
Antisera			
87-012	**2560**	160	<10
174F	160	**160**	<10
145SL	40	40	**640**

PRNT (plaque reduction neutralization test) data, preliminary results. Rabbit sera against TEMV and EMCV with a titer of 1:160 homologous had a titer less than 10 to the three isolates. Several attempts to make antisera with neutralizing titer in bank voles, mice, rabbits and guinea pigs have failed. All animals made high titer antibodies by IFT but no by PRNT. Bank voles failed to make IFT antibodies.

Sequence data

Sequences from 5'UTR and polyprotein gene of Ljungan virus isolates.

Cardiovirus consensus primers yielded a product of 303 bp for the three isolates 87-012, 174F and 145SL, compared to 284 bp for EMC virus. The fragment amplified was located immediately after the end of the poly C tract in EMC virus. PCR products specific for the Ljungan isolates were only obtained when the reannealing temperature was 50°C, and not at 58°C, which was optimal for

obtaining products from EMC virus cDNA. The subsequent sequence analysis revealed that the ATVEM primer was mismatched at 4 internal positions, explaining this difference in reannealing temperature. An alignment of the 5'UTR sequences for the three Ljungan isolates, EMCV and Vilyuisk virus (Table 2) shows a greater similarity between EMCV and Vilyuisk virus than between either of the two and the Ljungan isolates. It also demonstrates that each Ljungan isolate is distinct from the other by a number of nucleotide changes. The 174F and 145SL are similar to the isolate 87-012. The sequence homology between 174F and 87-012 was at most 95% (three undetermined bases in the sequence) while the homology between 87-012 and 145SL was 91%.

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The strategy chosen for obtaining additional PCR fragments from the Ljungan virus isolates was a modification of a technique for detecting differentially expressed mRNAs (Bauer, D., et al. 1993. Nucl. Acids Res. 21:4272-4280). As a test for this strategy, cDNA from the Ljungan 145SL isolate was amplified using the conditions above, using either the SLJU1 or the SLJU2 primer as a sense primer and one of twenty 10-mer oligonucleotides of randomly chosen sequence as "antisense" primer.

Sub. B3

If the PCR products obtained with the SLJU1 or SLJU2 primers and a specific 10-mer were similarly sized, and none of the primers yielded a product of this size when used alone in the PCR reaction, the fragment obtained was isolated and cloned. Only one combination of primers satisfied this criterion, namely the SLJU1 or SLJU2 primers in combination with the 118 10-mer oligonucleotide, which yielded a 1.8-1.9 kb PCR product. Of this fragment, 819 bp were sequenced from the 3' end. This sequence contained an open reading frame (ORF) of 663 bp in the sense of the viral polyprotein. This ORF was used to search in the Swiss protein data bank using the BLITZ search service from EMBL with the default search parameters. The top 10 scores were picornavirus polyprotein sequences, including 8 cardiovirus sequences. Homology was found over 188 a.a. The relatedness of this segment of the viral polyprotein to previously sequenced cardioviruses is shown in Table 3. A comparative

Sub. B3

alignment of all cardioviruses was made available to us by Dr. A. Palmenberg. In Table 3, the sequence of TMEBeAn was arbitrarily taken as the index strain. For the 12 remaining cardioviruses in the alignment, only differences in amino acid sequence are shown. The alignment of the Ljungan 145SL sequence is similarly represented at the top. Since the BLITZ search algorithm takes into account identical as well as similar amino acids, the latter have been indicated by small type, while differences to TMEBeAn is in capitals as for the other strains in the alignment.

In conclusion, the above presented data for the Ljungan isolates are characteristic for the 3 viruses but yet incomplete. However, the comparison of cloned sequences from both a highly conserved part of the 5'-untranslated region of cardioviruses and coding sequences for the viral capsid proteins of one isolate (Ljungan 145SL) clearly show that the Ljungan viruses are related to the cardioviruses, but are more distant relatives than any previously identified cardiovirus. While the amino acid homology (identical amino acids) of the viruses within the Theiler group is 96-97%, the homology to Vilyuisk virus is about 83%, and the EMC viruses are 67-74% homologous to TMEBeAn, the Ljungan 145SL has only about 32% identical amino acids to TMEBeAn. Even if homology is taken as identical and similar amino acids, this measure of relationship would still amount to only 50% between Ljungan 145SL and TMEBeAn (the corresponding figure would be 79-83% between EMC and TMEBeAn).

ALIGNMENT OF SEQUENCES

Sub. B4

Table 2 shows an alignment of three Ljungan virus isolates (1. 87-012, 2. 174F, 3. 145SL)[SEQ ID NO: 1,2 and 3, respectively] with published cardiovirus sequences (4. TMEBeAn, 5. Vilyuisk, 6. EMCV). The aligned sequence starts 29 nt 3' of the end of the poly-C tract in EMCV, and the sequence corresponds to nt 557 - 808 (approximately) in the different viral genomes. Inserted spaces in the sequences are indicated by a period (.).

TABLE 2

1. AGTCTAGTCTTATCTTGTATGTGTCCTGCACT..GA..ACTTGTTTCTGT
2. AGTCTAGTTTCATTCTGTGTGTGTTTGGCACT..GA..AATTATTTCTGT
3. AGTTTGGTTCTCTCTTGAGTGTGTTTTGTGTT..AG..CATAATTTCTGT
4. TGACAGG.GTTATTTTCACC.TCTTCTT..TTCTACTCCACAG.TG.T.T
5. TGACAGG.GTTATTTTCACC.TCTTCTCTCTTCTACTTCATAG.TG.T.T
6. AGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCAC..CATA.TTGCCGT

1. CTCTGGAGTGCTCTACACTTCAGTAGGGGCTGT.A.CCCGGGCGGTCCCA
2. CTCTGGGGTGCTTTACACTTCAGTAGGGGCTGT.A.CCCGGGCGGTCCCA
3. CTCTAGAGTGCTTTACACTCTAGTAGGGGCTGT.A.CCCGGGCGGTCCCA
4. CT.A.....TACTGTG..GAAGGGTATGTGT....TGCCCCTTCCT
5. CT.A.....TACTATG.AA.AGGGTATGTGT..C..GCCCCTTCCT
6. CT.T.....TTGGCAATGT.G.AGGGCCCG.GAAACCTGGCCCTGTCT

1. CTCTTCACAGGAATCTGCACAGGTGGCTTTCAC.CTCTGGACAGTGCATT
2. CTCTTCACAGGAATNTGCACAGGTGGCTTTCAC.CTCTGGACAGTGCATT
3. CTCTTCACAGGAATCTGCACAGGTGGCTTTCAC.CTCTGGACAGTGCATT
4. .TCTTGGAGAACGT..GCGCGGCGGTCTTTCCGTCTCTCGACAA.GCGC.
5. .TCTTGGAGAACGT..GCGTGGCGGTCTTTCCGTCTCTCGAAAAACG..T
6. .TCTTGACGAGCAT.T.CCTAGGGGTCTTTCCC.CTCTCGCCAAAGGAAT

1. CCACACCCG.C.TCCACGGTAGAAGATGATGTGTGTCTTTGCT..TGTGA
2. CCACACCCG.C.TCCACAGTAGAAGATGATGTGTGTCTTTGCT..TGTGA
3. CCATACCCG.C.TCCACAATAGAAGATGATGTATATCTTTGTT..TGTGA
4. GCGT..GCAACATACAGAGT.AACG.CGAAGAA.AGCA..GTTC.TC.GG
5. GCGT..GCGACATGCAGAGT.AACG.CAAAGAA.AGCA..GTTC.T.TGG
6. GCA.A.G.GTC.TGTTGAAT.GTCG.TGAAGGA.AGCA..GTTCCTCTGG

1. AAA.GCTT...GTGAAAATC.....GTGTGTAGGCGTAGCGGCTACT
2. AAA.GCTT...GTGAAAATC.....GTGTGTAGGCGTAGCGGNTACT
3. AAT.GCT.CA..TGAA.A.C.....GTGTGTGTAGGCGTAGCGGCTACT
4. TCTAGCT.CTAGTGCCCA.CAAGAAAACAGCTGTAG.CG.ACCA.C.ACA
5. TCTAGCT.CTGGTGCCCA.CAAGAAAACAGCTGTAG.CG.ACCA.C.ACA
6. AA..GCTTCT..TGAAGA.CAA.ACAACGTCTGTAG.CG.ACC..CT..T

1. TGAGTGCCAGCGGATTACCCCTAGTGGTAACACTAGC
2. TGAGTGCCAGCGGACNACCCCTAGTGGTAACACTAGC
3. TGAATGCCAGCGGAACCCCCCTAGTGGTAACACTAGC
4. ..AAGGC.AGCGGAACCCCCCTCCTGGTAACAGGAGC
5. ..AAGGC.AGCGGAAACCCCCTCCTGGTAACAGGAGC
6. TGCAGGC.AGCGGAACCCCCCACCTGGCGACAGGTGC

In this region of the viral genome, Ljungan 174F has 94% homology to Ljungan 87-012 (here taken as the indicator strain for comparisons), and Ljungan 145SL

has 91% homologous residues to Ljungan 87-012. The TMEBeAn strain has 69%, Vilyuisk has 68% and EMCV has 68% homologous residues to Ljungan 87-012. Using the same criteria for calculating the homology, EMCV has 85% homology to TMEBeAn.

Table 3 shows alignment of cDNA sequences from the polyprotein coding sequences of the Ljungan 145SL isolate [SEQ ID NO. 4] to the amino acid sequences of sequenced cardioviruses in the comparative alignment compiled by Dr. A. Palmenberg (personal comm.) The TMEBeAn strain was arbitrarily taken as the indicator strain, while the amino acids of the remaining strains are shown only if they differ from the indicator strain. For the Ljungan 145SL isolate, similar, but non-identical amino acids are indicated in small type. The amino acid homology between Ljungan 145SL and other cardioviruses was established screening the entire Swiss Protein Data Bank using the BLITZ search algorithm with standard search parameters.

TABLE 3

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Ljungan 464              480                                              525
145SL   K--m-iArm-sVyK-ERTEPGGTNG--QWshtHSPInW-.fDGGiHLED-P-..-LFsSCy-
TMEBeAn SDLLELCKLPT.FLGNPNTNNKRYPYFSATNSVPATSMVDYQVALSCSCMANSMLAAVARNFN
 TMEGd7 ----------.-----S-D----------------L-------------------------
 TMEGd7 ----------.-----S-D----------------L-------------------------
  TMEDa ----------.------S---------------T--L-----------C------------
Vilyuisk T----------.----L.S-DT-V-F-T------TE-L-E---T------S-----S------
   EMCBd K-F--IAQI--.-I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L--TF---LS---A
   EMCBc K-F--IAQI--.-I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L--TF---LS---A
   EMCDd K-F--IAQI--.-I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L--TF---LS---A
   EMCDc K-F--IAQI--.-I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L--TF---LS---A
  EMCDv1 K-F--IAQI--.-I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L--TF---LS---A
    EMCR K-F--IAQI--.-I--KIP-AVP-IEA-N-.A-KTQPLAT---T-----L--TF---LS---A
  MengoM K-F--IAQI--.-I--KMP-AVP-IEA-N-.A-KTQPLAV---T-----L--TF---LS---A
Mengo37a K-F--IAQI--.-I--KVP-AVP-IEA-N-.A-KTQPLAV---T-----L--TF---LS---A
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Ljungan 526          540                                              588
145SL   Yw---TVLKLTVYAsTFN--rLRm-fF-I.MMqG-Q-.kKHkCLfMvC-i---nt-EM-I-y.
TMEBeAn QYRGSLNFLFVFTGAAMVKGKFLIAYTPPGAGKPTTRDQAMQSTYAIWDLGLNSSFNFTAPFI
 TMEGd7 ----------------------R-----------------A--------------------
TMEGd7b ---------------------------------------A--------------------
  TMEDa ---------------------------------------A-------------V------
Vilyuisk --------------S--T----------------------X-----------------V---
   EMCBd ------VYT-----T--M-----------------S------A-----------YS--V---
   EMCBc ------VYT-----T--M-----------------S------A-----------YS--V---
   EMCDd ------VYT-----T--M-----------------S------A-----------YS--V---
   EMCDc ------VYT-----T--M-----------------S------A-----------YS--V---
  EMCDv1 ------VYT-----T--M-----------------S------A-----------YS--V---
    EMCR ------VYT-----T--M-----------------S------A-----------YS--V---
  MengoM ------VYT-----T--M-----------------S------A-----------YS--V---
Mengo37a ------VYT-----T--M-----------------S------A-----------YS--V---
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Ljungan 589       600                                                   651
145SL   ..-.w..GnwMR--RG--I--lRiDV-NR---N-Ss-NAVnCiLQ-KM-n-AKFMv-TT-NIV-
TMEBeAn SPTHYRQTSYTSPTITSVDGWVTVWKLTPLTYPSGTPTNSDILTLVSAGDDFTLRMP.ISPTKW
 TMEGd7 ------------------------Q-------------------------------.------
TMEGd7b ------------------------Q-------------------------------.------
  TMEDa ------------A--A---------Q---------A-V------------------.------
Vilyuisk --S----------S-AA----L---Q-----F-ANV-PS----------N-------.------
   EMCBd ----F-MVGTDQVN--N--------Q-------P-C--SAK---M-----K--S-K--.---AP-
   EMCBc ----F-MVGTDQ------------Q-------------------------------.------
   EMCDd ----F-MVGTDQ------------Q-------------------------------.------
   EMCDc ----F-MVGTDQ------------Q-------------------------------.------
  EMCDv1 ----F-MVGTDQ------------Q-------------------------------.------
    EMCR ----F-MVGTDQ-----A------Q-------------------------------.------
  MengoM ----F-MVGTDQA-----------Q-------------------------------.------
Mengo37a ----F-MVGTDL-----A------Q-------------------------------.------
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Serological assay indicating relationship between the Ljungan viruses and diabetes mellitus and myocarditis.

A serological assay using indirect immunofluorescense test using virus infected acetone fixed green monkey kidney cells was established. Patient sera were screened at a 1:8 dilution and positive sera titrated. Sera with a titer of 1:32 or more were considered positive.

Sera from 59 children (age 1-16) from the Stockholm area recently diagnosed with Diabetes Mellitus (DM) and 34 control children from the same geographic area, were tested for presence of antibodies to the three viruses of the invention. Nineteen of the 59 (32%) DM patients screened positive and 2 of the 34 (6%) controls were found positive to one or more of the 3 viruses (significant difference p=0.002, Fisher´s exact test). Nine recently diagnosed DM patients (age 23-46) from Medelpad county were also tested. Two controls were selected for each adult DM patient and they were matched for age, sex and geographic area of residence. Five of the nine (56%) DM patients and one of the 18 (6%) control patients were found positive to one or more of the 3 viruses (significant difference p=0.008 Fisher´s exact test).

Serum was also available from 5 athletes dying suddenly in myocarditis. Three controls were selected for each myocarditis patient and they were matched for age, sex and geographic area of residence. Four of the 5 (80%) patients dying from myocarditis and 1 of the 15 (7%) controls where found positive to one or more of the three Ljungan viruses (significant difference p=0.005, Fisher´s exact test).

DESCRIPTION OF DIFFERENT ASPECTS OF THE INVENTION

In the following, different aspects of the invention will be disclosed. However, all of these aspects are related to a new group of picornaviruses.

Thus, a first aspect of the invention is directed to a new group of picornaviruses, namely picornaviruses comprising in their viral genome, more precisely in the non-coding region, a nucleotide sequence corresponding to a cDNA sequence selected from the group consisting of

SEQ ID NO: 1 (Ljungan 87-012)

AGTCTAGTCT TATCTTGTAT GTGTCCTGCA CTGAACTTGT TTCTGTCTCT 50
GGAGTGCTCT ACACTTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100
ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC 150
CGCTCCACGG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA 200
AAATCGTGTG TAGGCGTAGC GGCTACTTGA GTGCCAGCGG ATTACCCCTA 250
GTGGTAACAC TAGC

and homologous sequences having at least 75 % homology to the SEQ ID NO: 1. The picornaviruses of the invention should further cause mammalean disease.

In a preferred embodiment of this aspect of the invention said homologous sequences have at least 80%, at least 85% or at least 90% homology to the SEQ ID NO: 1.

In a particularly preferred embodiment said homologous sequence is one of

SEQ ID NO: 2 (Ljungan 174F)

AGTCTAGTTT CATTCTGTGT GTGTTTGGCA CTGAAATTAT TTCTGTCTCT 50
GGGGTGCTTT ACACTTCAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100
ACAGGAATNT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCACACC 150
CGCTCCACAG TAGAAGATGA TGTGTGTCTT TGCTTGTGAA AAGCTTGTGA 200
AAATCGTGTG TAGGCGTAGC GGNTACTTGA GTGCCAGCGG ACNACCCCTA 250
GTGGTAACAC TAGC

and

SEQ ID NO:3 (Ljungan 145SL).

AGTTTGGTTC TCTCTTGAGT GTGTTTTGTG TTAGCATAAT TTCTGTCTCT 50
AGAGTGCTTT ACACTCTAGT AGGGGCTGTA CCCGGGCGGT CCCACTCTTC 100
ACAGGAATCT GCACAGGTGG CTTTCACCTC TGGACAGTGC ATTCCATACC 150
CGCTCCACAA TAGAAGATGA TGTATATCTT TGTTTGTGAA ATGCTCATGA 200
AACGTGTGTG TAGGCGTAGC GGCTACTTGA ATGCCAGCGG AACCCCCCTA 250
GTGGTAACAC TAGC.

These sequences (ID NO: 2 and 3) have 94% homology and 91% homology to the SEQ ID NO: 1, respectively.

It should be understood that homologies in the coding region of different viruses of the invention may vary considerably, but in the non-coding region they share a homology of at least 75% with the SEQ ID NO: 1.

The nucleotide sequences, SEQ ID NO: 1, 2 and 3, correspond to approximately nucleotides 557 - 808 (a conserved region) in the genome of encephalomyelitis virus (EMCV). These three viruses have been isolated from wild rodents, more precisely bank voles. The viruses can be multiplied in cell lines, and for a large-scale production of picornavirus products the virus genome can be inserted into other microorganisms.

A second aspect of the invention is directed to a protein comprising an amino acid sequence selected from the group consisting of

SEQ ID NO: 4 (partial structural protein of Ljungan 145)

Lys Asp Leu Met Glu Ile Ala Arg Met Pro Ser Val Tyr Lys Gly Glu
5 10 15
Arg Thr Glu Pro Gly Gly Thr Asn Gly Tyr Phe Gln Trp Ser His Thr
20 25 30
His Ser Pro Ile Asn Trp Val Phe Asp Gly Gly Ile His Leu Glu Asp
35 40 45
Met Pro Asn Leu Asn Leu Phe Ser Ser Cys Tyr Asn Tyr Trp Arg Gly
50 55 60
Ser Thr Val Leu Lys Leu Thr Val Tyr Ala Ser Thr Phe Asn Lys Gly
65 70 75 80
Arg Leu Arg Met Ala Phe Phe Pro Ile Met Met Gln Gly Thr Gln Arg
85 90 95
Lys Lys His Lys Cys Leu Phe Met Val Cys Asp Ile Gly Leu Asn Asn
100 105 110
Thr Phe Glu Met Thr Ile Pro Tyr Thr Trp Gly Asn Trp Met Arg Pro
115 120 125
Thr Arg Gly Ser Val Ile Gly Trp Leu Arg Ile Asp Val Leu Asn Arg
130 135 140
Leu Thr Tyr Asn Ser Ser Ser Pro Asn Ala Val Asn Cys Ile Leu Gln
145 150 155 160
Val Lys Met Gly Asn Asp Ala Lys Phe Met Val Pro Thr Thr Ser Asn
165 170 175
Ile Val Trp ,

and homologous sequences having at least 75% homology to the SEQ ID NO: 4,

and antigenic fragments of the sequences.

In an embodiment of the invention the homologous sequences have at least 80%, at least 85% or at least 90% homology to the SEQ ID NO: 4.

The SEQ ID NO: 4 is the result of preliminary partial sequencing of the cDNA sequence from the polyprotein coding sequence of the virus Ljungan 145 SL isolate. Said protein comprising said amino acid sequence SEQ ID NO: 4, said homologous sequences and said antigenic fragments are useful as active ingredients in medicines and as diagnostic reagents in diagnostic kits.

A third aspect of the invention concerns an antiserum or antibody directed against a structural protein of the virus defined in the first aspect of the invention. An example of such a structural protein is defined in the second aspect of the invention. Such an antiserum or antibody is useful as an active ingredient in medicines and as diagnostic reagent in diagnostic kits. Both polyclonal and monoclonal antibodies may be used, and these are suitably produced by using said virus or fragments thereof specific for said virus for immunizing mammals.

A fourth aspect of the invention is directed to an antigen comprising at least a part of a structural protein of the picornavirus defined in the first aspect of the invention, including a subunit thereof. An example of such an antigen is the protein and antigenic parts thereof defined in the second aspect of the invention. Such an antigen of the invention is useful as an active ingredient in medicines and as a diagnostic reagent in diagnostic kits.

A fifth aspect of the invention is directed to a diagnostic kit comprising at least one member from the group consisting of

a) an antiserum or antibody according to the third aspect of the invention or an antigen-binding part thereof,

b) an antigen according to the fourth aspect of the invention or an antibody-binding part thereof,

c) one or several probes designed with respect to the genome of the virus according to the first aspect of the invention,

and

d) one or several primers designed with respect to the genome of the virus according to the first aspect of the invention.

The different members of a diagnostic kit will depend on the actual diagnostic method to be used. In addition to the above-listed possible members of the diagnostic kit, said kit may contain positive reference samples, negative

reference samples, diluents, washing solutions and buffers as appropriate. The kit will further be accompanied by instructions for use.

The above-listed members a) and b) find use in immunodiagnostic methods, such as enzyme-liked immunosorbent assay (ELISA), radioimmunoassay (RIA) or immunofluorescence assay (IFA).

The above-listed members c) and d) find use in direct virus detection. Preferably, a diagnostic method based on the PCR (polymer chain reaction) technique with such primers is utilized in the direct detection of a virus according to the invention.

All of the above mentioned diagnostic methods are well known in the art, and a man of ordinary skill in the art will readily select useful members for a diagnostic kit in relation to the diagnostic method to be used.

A sixth aspect of the invention relates to a vaccine having as an immunizing or neutralizing component a member selected from the group consisting of
a) the virus according to the first aspect of the invention,
b) the virus according to the first aspect of the invention in attenuated form,
c) the virus according to the first aspect of the invention in killed form,
d) an antigen according to the fourth aspect of the invention, including a subunit of the virus according to the first aspect of the invention,
and
e) DNA corresponding to the genomic RNA of the virus according to the first aspect of the invention.
In an embodiment of this aspect of the invention said vaccine may additionally comprises an adjuvant. Such an adjuvant must of course be an adjuvant which is approved for use in vaccines by authorities responsible for veterinary or human medicines.

The vaccine may contain other ingredients which are needed for specific preparations intended for oral, subcutaneous, intramuscular or intradermal administration. Suitable additional ingredients are disclosed in the European or US Pharmacopoeia.

The alternative members a), b) and c) are all examples of conventional whole virus, attenuated virus, and subunit vaccines developed for other types of viruses, and the member d) represents DNA incorporation into body-specific cells, which then will express virus-specific structures and elicit immunity against said virus.

A seventh aspect of the invention is directed to a picornavirus according to the first aspect of the invention, optionally in attenuated or killed form, an antiserum or antibody according to the third aspect of the invention or an antigen according to the fourth aspect of the invention, for use in a medicament (for veterinary or human use). An example of such a medicament is a vaccine according to the invention disclosed in the sixth aspect thereof.

The eight aspect of the invention concerns use of a picornavirus according to the first aspect of the invention, optionally in attenuated or killed form, an antiserum or antibody according to the third aspect of the invention or an antigen according to the fourth aspect of the invention, in the preparation of a medicament for prophylactic or therapeutic treatment of a disease caused by said virus.

In an embodiment of said use the disease caused by said virus is one of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

A ninth aspect of the invention is directed to a method of prophylactic or therapeutic treatment of a disease caused by a virus according to the first aspect of the invention in a mammal, including human, which comprises administering to said mammal a prophylactically or therapeutically effective amount of a medicament comprising as an active ingredient a member of the group consisting of

a) the virus according to the first aspect of the invention,

b) the virus according to the first aspect of the invention in attenuated form,

c) the virus according to the first aspect of the invention in killed form,

d) an antigen according to the fourth aspect of the invention, including a subunit of the virus according to the first aspect of the invention,

and

e) DNA corresponding to the genomic RNA of the virus according to the first aspect of the invention.

In an embodiment of said method the disease caused by said virus is one of Myocarditis, Cardiomyopathia, Guillain Barré Syndrome, and Diabetes Mellitus, Multiple Sclerosis, Chronic Fatigue Syndrome, Myasthenia Gravis, Amyothrophic Lateral Sclerosis, Dermatomyositis, Polymyositis, Spontaneous Abortion, and Sudden Infant Death Syndrome.

The actual dosage regimen will be determined by the vaccine producer based on animal experiments and clinical trials.

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